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Sperm Quality and Ascorbic Acid Concentration in Rainbow Trout Semen Are Affected by Dietary Vitamin C: An Across-Season Study[1]

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ABSTRACT

High concentrations of ascorbic acid occur in scurvy-prone, teleost fish seminal plasma. We quantified seasonal relationships between 1) dietary level of vitamin C and level of seminal plasma ascorbic acid, and 2) seminal plasma ascorbic acid concentration and sperm quality, in rainbow trout (*Oncorhynchus mykiss*). We maintained six groups of 2-yr-old rainbow trout on diets supplemented with 0, 30, 110, 220, 440, and 870 ppm ascorbyl monophosphate beginning in May 1992. Sperm were produced during the end of October 1992 through April 1993; we collected milt 13 times from 3–8 fish per treatment. We quantified ascorbic acid concentration in seminal plasma, and sperm concentration, motility, and weight. Seminal plasma ascorbic acid concentrations were affected directly by the ascorbyl monophosphate level in the diet. Seminal plasma ascorbic acid concentrations also were affected by season. Ascorbic acid deficiency did not influence semen quality (sperm concentration and motility) at the beginning of the spawning season. However, sperm concentration and motility in a group fed an ascorbic acid-free diet declined during the period of study. Ascorbic acid deficiency reduced both sperm concentration and motility, and thus fertility, of rainbow trout. These results indicate that vitamin C is important for male fish reproduction; the dietary requirement for seminal plasma ascorbic acid saturation exceeds that for optimum growth.

INTRODUCTION

Teleost fishes, like humans, nonhuman primates, bats, and guinea pigs, do not have an ascorbic acid (AA)-synthesizing capacity, for they lack gulonolactone oxidase [1, 2]. Thus, they depend on exogenous sources. This is recognized in aquaculture, where vitamin C is currently added to fish food. The positive relationship between the AA level in fish tissues and the dietary AA level has been documented in numerous works [3–7].

The role of AA in fish reproduction has been extensively studied in females. High concentrations of AA in fish ovaries have been reported, and an AA role in steroidogenesis has been postulated [7, 8]. The fixation of radiolabeled AA in rainbow trout egg membranes has been demonstrated [9]. Vitamin C increases egg hatchability; the vitamin pool in eggs can be transferred to newly hatched fry to provide vitamin C during early life [8, 10]. However, no data exist on the function of AA in the reproductive system of male fish.

AA is one of the main reducing agents of low molecular weight in human seminal plasma [11]. The level of AA can be changed by dietary treatment, and the beneficial effect of vitamin C on human sperm physiology has been reported, by Dawson et al. [12]. These authors found an improvement in many sperm qualities, such as viability, motility, agglutination, amount of sperm precursors, and morphology, following one week of AA supplementation. By using vitamin C supplementation therapy, it was possible to improve the sperm quality of smokers [13]. Severe histological damage and decline in sperm count and motility as well as an increase in number of abnormal sperm have been reported for guinea pigs placed on a scorbutic diet [14].

Our previous work [15] indicated much higher concentrations of vitamin C in fish seminal plasma (30–60 ppm) than in blood plasma (1–10 ppm), suggesting that AA may be important in male fish. Our study sought to determine whether dietary AA can influence 1) rainbow trout seminal plasma vitamin C concentrations and 2) milt weight and sperm concentration and motility.

MATERIALS AND METHODS

Experimental Design and Source of Milt

Two-year-old rainbow trout (golden variety of London, Ohio, strain) were maintained at a density of 11 fish (mean male weight ± SEM: 245 ± 85) per tank in six 750-L indoor fiberglass tanks. Males and females were stocked together; there were 40 males and 26 females at the beginning of the experiment. Each tank was supplied with a constant flow of well water. Water temperature followed the natural course, decreasing from 12°C in November to about 8°C by the end of January and then increasing again to 12° in April. Fish

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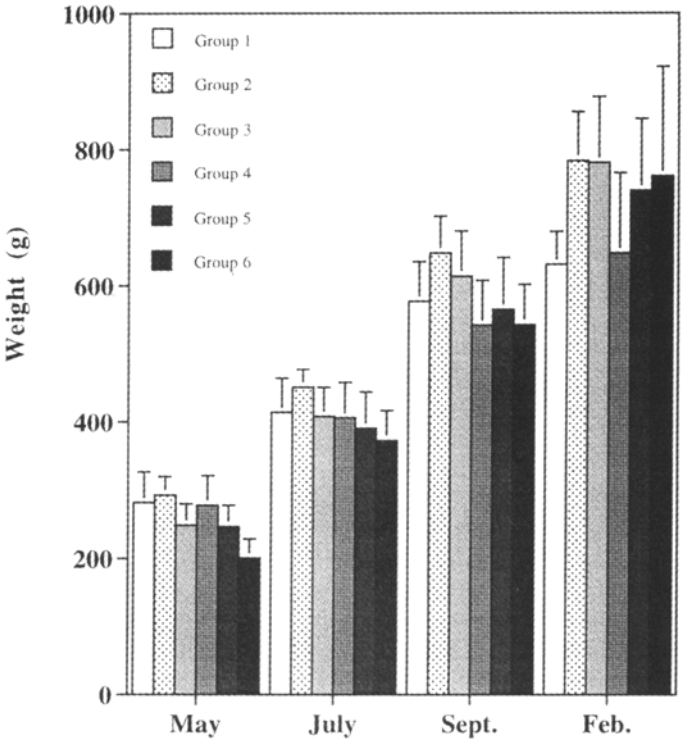

FIG. 1. Growth in wet weight of rainbow trout during May through February. Fish diets were supplemented with 0, 30, 110, 220, 440, and 870 ppm ascorbyl monophosphate for groups 1, 2, 3, 4, 5, and 6, respectively. Data are presented as mean ± SEM. Differences between effects of dietary treatments on body weights within months were not significant.

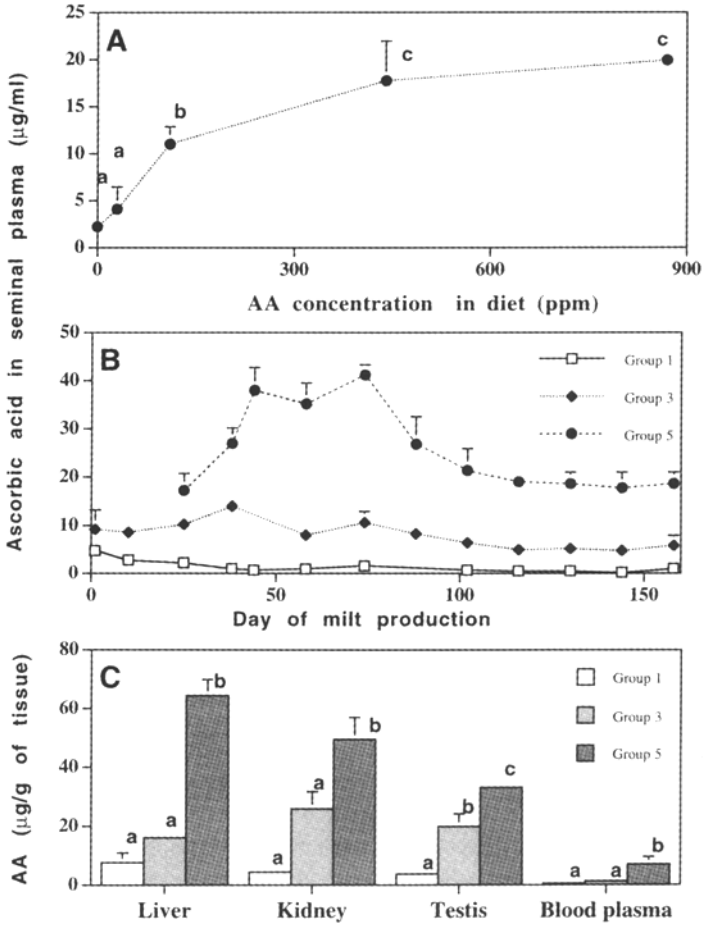

FIG. 2. Effect of dietary AA monophosphate on AA concentrations in rainbow trout: A) AA in seminal plasma at Day 25; N = 6, 2, 7, 2, 3 for groups 1, 2, 3, 5, and 6, respectively. B) seasonal changes in AA in seminal plasma of fish fed the same diet supplemented with 0 (group 1), 110 (group 3), and 440 (group 5) ppm ascorbyl monophosphate; N = 2–8 per group. C) AA in fish organs and blood plasma (μg/ml) at end of reproductive season; N = 3. Data are presented as mean ± SEM. Different letters indicate significant differences ($p < 0.05$) among groups.

in groups 1–6 were fed twice daily (1% body weight per day) with fish meal-based diets [8] supplemented with 0, 30, 110, 220, 440, and 870 ppm ascorbyl phosphate (AP) magnesium salt (Showa Denko America Co., New York, NY), respectively. Monophosphate ascorbyl ester was used because of its resistance to heat and diet processing in comparison to unprotected AA and its excellent availability to fish [2]. Ascorbyl ester was analyzed in the diet offered to fish by HPLC [16].

Feeding began in May 1992. Milt collection began 26 October 1992, Day 1 of the sperm production season as defined by Baynes and Scott [17], and ended in early April 1993. Fish were stripped 13 times during October through April. The average frequency of stripping was 13 ± 2 days. Before semen collection, fish were anesthetized with tricaine (MS-222; Argent, Redmond, WA). We sampled all available semen each time. On 15 April, three fish from each group were killed for tissue (liver, kidney, testis) AA analysis. Some fish did not spermiate at all or occasionally produced only minute amounts of milt (28% of males). We were not able to collect milt from these fish throughout the whole season because their milt production was asynchronous and occurred during a shorter period. The frequency of such disturbances was not related to dietary treatment; they occurred mainly in groups 2, 4, and 6. A similar variation in the maturation of rainbow trout males has also been reported by other authors [17–21]. Only groups 1, 3, and 5 provided sufficient samples (at least three observations per treatment) for across-season comparisons. For this reason, seasonal effects were examined exclusively for these groups. Data from all groups were used 1) to test the effect of dietary vitamin C on fish growth by one-way ANOVA (Fig. 1), 2) to determine the relationship between AA concentration in the diet and in seminal plasma at Day 25 (Fig. 2A; data available for groups 1, 2, 3, 5, 6), and 3) for correlation analysis.

Sperm Characterization

Sperm concentration was measured by a spectrophotometric technique as described previously [22]. Sperm motility was estimated by microscopic observation of milt diluted 100-fold in a solution consisting of 125 mM NaCl, 20

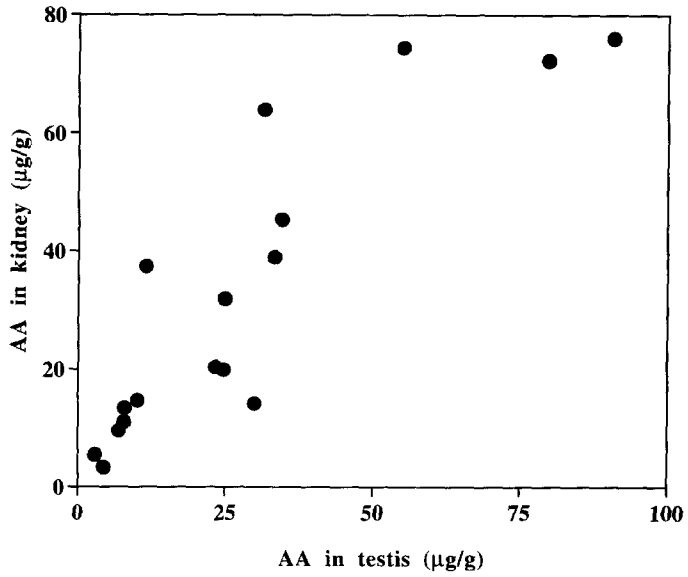

FIG. 3. Relationship between AA concentrations in testis and in kidney of rainbow trout. Data were obtained in April at end of reproductive season.

mM Tris, and 30 mM glycine, pH 9.0 [23]. Milt was weighed to the nearest 0.1 g.

Milt was centrifuged at 10 000 × *g* after 2–4-h storage on ice; trichloroacetic acid extracts for AA measurement were prepared from fresh seminal plasma. Extracts were frozen for further analysis at −20°C. Total AA and dehydroascorbic acid (DHA) concentrations in fish tissues and seminal plasma were determined according to the 2,4-dinitrophenylhydrazine colorimetric method of Roe and Kuether [24] with modifications described by Dabrowski and Hinterleitner [25].

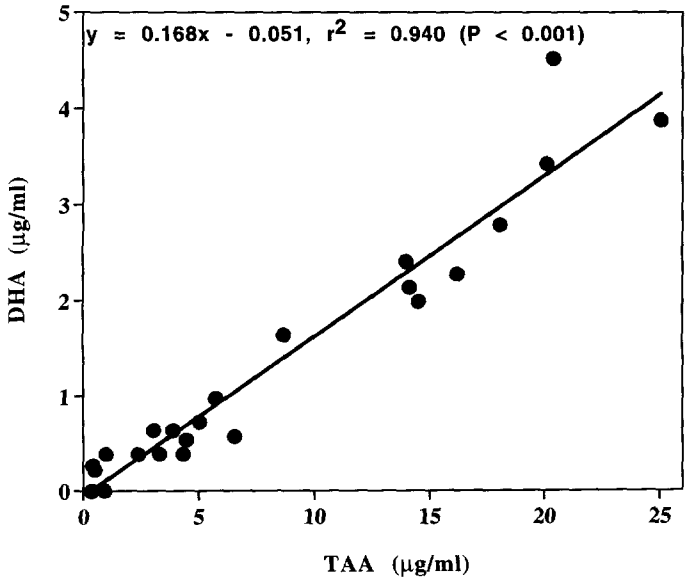

FIG. 4. Relationship between concentration of TAA and DHA in seminal plasma at Day 116 of milt production.

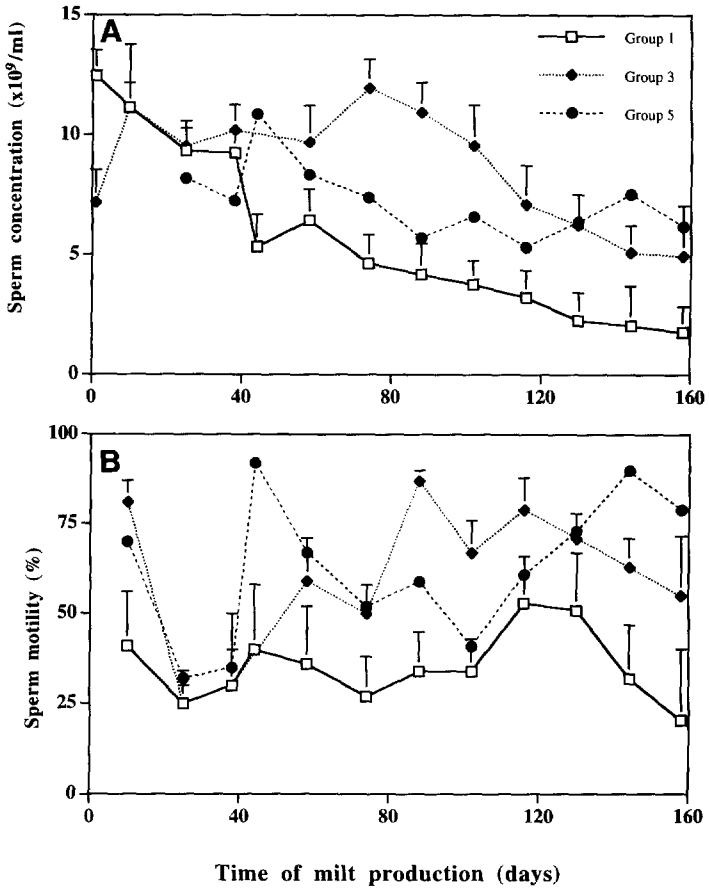

FIG. 5. Evolution of sperm concentration (A) and motility (B) during reproductive season of rainbow trout in relation to different dietary treatments. Ascorbyl monophosphate concentrations were 0, 110, and 440 ppm in groups 1, 3, and 5, respectively. Data are presented as mean ± SEM.

Statistical Analysis

We used analysis of variance (ANOVA) for analysis of differences between groups across season. Dependent variables were milt weight, sperm concentration and motility, and total AA (TAA) concentration in seminal plasma; independent variables were days of sperm production and dietary treatments. Repeated milt productions (from the same males) were included in the analysis. Because of the lack of data for all three groups for Days 1, 10, 25, 44, and 116, only results for Days 38, 58, 74, 88, 102, 130, 144, and 158 were included in analyses of sperm motility and concentration. Data of Day 25 were additionally available for seminal plasma AA analysis. A significant interaction occurred between day and treatment for sperm concentration ($p < 0.01$) and TAA concentration in seminal plasma ($p < 0.01$); however, there was no interaction in the case of motility ($p < 0.59$). Even though there were significant interactions, taking into consideration the nature of the interaction (concordant), we can generalize relative to the interpretation of the contrast. We used PROC GLM in the SAS Data Analysis Package (SAS Institute Inc., Cary, NC) to analyze group × season comparisons. The statistical package StatView (Ab-

acus Concepts, Inc., CA) was applied in correlation calculations, and one-way analysis of variance was used to compare body weights and AA concentrations in seminal plasma at Day 25 of the sperm production season, and in tissues (see *Results*: Fig. 1, Fig. 2, A and C).

RESULTS

Diet analysis revealed that 73.6 ± 6.1% of ascorbyl monophosphate was retained 1 yr after diet processing and cold storage. AA content in this preparation was 43.3%. Dietary supplementation of AA thus corresponded to 0, 10.4, 17.5, 76.8, 129.3, and 270.9 mg AA/kg. No significant effect of dietary vitamin C on fish growth occurred (Fig. 1) nor were scurvy symptoms (malformation of gill opercula lordosis [5]) observed during this period. For the first time, we documented that AA concentration in fish seminal plasma was affected directly by dietary vitamin C (Fig. 2A). Supplementation of diets with 870 ppm AP resulted in maximal concentration of vitamin C in seminal plasma; values obtained with 440 ppm AP in the diet were close to saturation level.

Both effects of season and group on seminal AA concentrations were significant ($p < 0.05$). The values of group 5 were higher than those of groups 1 and 3, and those of group 3 were higher than that of group 1 for day 38; the differences for Days 25, 58, and 74 were significant at $p = 0.051–0.06$. Contrast analysis revealed significant differences between AA-deficient group 1 and combined AA-supplemented groups 3 and 5. Vitamin concentrations declined towards the end of the reproductive season in groups 1 (nonsignificant differences) and 3 (significant differences between data from the beginning and the end of the season). Group 5 had peak values between Days 38 and 74 of milt production, significantly higher than the values before (Day 25) and after this time (Fig. 2B). Higher concentrations of AA were recorded in seminal plasma during the first three strippings of the AA-free diet-fed group (4.74 ± 0.99, 2.78 ± 1.11, and 2.22 ± 0.82 ppm for Days 1, 10, and 25 of sperm production, respectively); however, the vitamin concentrations declined later below 1 ppm (Fig. 2B).

TAA concentrations in tissues of male rainbow trout correlated with the dietary level of AP (Fig. 2C). Gonadosomatic index (GSI) values amounted 0.84% ± 0.26, 0.77% ± 0.27, and 0.91% ± 0.39 for groups 1, 3, and 5, respectively. There were no significant differences in GSI between treatments. Testis concentration of AA significantly correlated with kidney and liver AA ($r = 0.87$ and 0.81 for kidney and liver, respectively; $p < 0.001$). In the kidney, which is involved in excretion of excess ascorbate, saturation was reached at AA concentrations of about 70 μg/g of tissue (Fig. 3).

DHA, which indicates vitamin C deficiency [5], amounted to 54.3% ± 6.8 of total seminal plasma AA in group 1 and was higher ($p < 0.01$) than in AA-supplemented groups

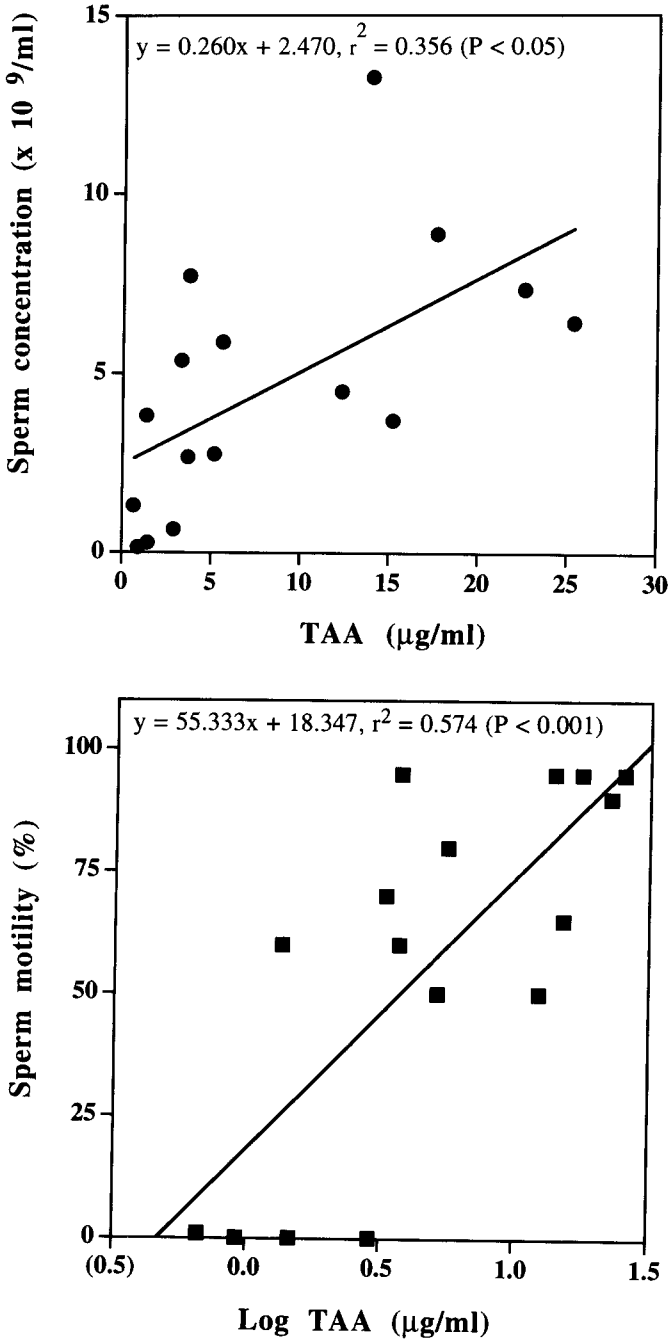

FIG. 6. Relationship between sperm motility and concentration, and seminal plasma AA at Day 158 of sperm production.

(16.4% ± 2.3 and 11.6% ± 1.3 for groups 3 and 5, respectively). The seminal levels of this form of vitamin C had significant correlation and linear relationship with total seminal AA levels (Fig. 4). Correlation coefficients ranged from 0.51 to 0.97 for pooled data of the same sampling days and were significant. No significant correlation occurred in 2 of 13 samplings.

Reduced sperm concentration was observed in the AA-free diet-fed group. (Fig. 5A; contrast analysis: group 1 vs. groups 3 and 5, $p < 0.05$). This effect did not occur until

about 40 days after the first sperm collection; we were not able to test data from the first sampling statistically. No differences in milt weight were observed; the average milt weights were 2.81 ± 0.27; 3.24 ± 0.35, and 3.14 ± 0.31 g per fish per sampling for groups 1, 3, and 5, respectively. For this reason, we conclude that differences in sperm concentrations were not caused by an increase in the seminal fluid secretion; thus, these differences may probably be attributed to an increase in spermatozoa produced during spermatogenesis. Sperm motilities of the AA-free diet treatment group were lower than those from fish fed with AA-supplemented diets (Fig. 5B; contrast analysis group 1 vs. the average of groups 3 and 5, $p < 0.001$). In the absence of a treatment-by-season interaction, it was possible to generalize regarding the treatment effect. As with the sperm concentration, no conclusion on the effect of AA on sperm motility at the beginning of the reproductive season could be made. The effect of season on sperm motility was significant ($p < 0.05$). Initial sperm motility was rather low at the beginning of the season, which agrees with our data for this broodstock (52.9 ± 28.1% [15]). Sperm concentration and motility were positively correlated with seminal plasma AA concentration (Fig. 6), but significant relationships occurred only for the last samplings. Motility was also correlated with seminal AA for Day 74 ($r = 0.47, p < 0.05$) and Day 144 ($r = 0.49, p < 0.05$), and significant correlations between seminal AA and sperm concentration were also found for Day 130 ($r = 0.46, p < 0.05$) and Day 144 ($r = 0.57, p < 0.01$).

DISCUSSION

We found that AA levels in seminal plasma are related directly to dietary concentrations of vitamin C. We also confirmed our earlier results that ascorbyl monophosphate can be an excellent source of vitamin C for rainbow trout [2]. This AA derivative is chemically stable and can be used by fish after dephosphorylation of AP to AA, primarily by intestinal alkaline phosphatase [26], given that only traces of AP are absorbed and occur in blood plasma [4]. For this reason, this ester can replace the very unstable dietary AA as a source of vitamin C in reproductively active fish. Although much lower dietary levels of AA are necessary to support normal fish growth (50 ppm, NRC 1993 [27]), our results suggest that 130–270 mg AA/kg resulted in AA concentrations in the seminal plasma of rainbow trout that were beneficial to sperm qualities. It should be emphasized that the currently recommended AA level does not achieve seminal plasma AA levels recorded in fish from natural environments; such levels were obtained only where diet was supplemented with 130–270 ppm AA [15].

Concentrations of AA in rainbow trout seminal plasma (30–60 mg/L) are comparable to those reported for mammalian seminal plasma (26–90 mg/L [11–13, 28]. However, in mammals, most of the seminal plasma AA pool is added to the spermatozoan environment from accessory glands (the seminal vesicle in humans) during ejaculation [11, 28]. For this reason, evaluation of the role of seminal AA in mammalian reproduction is difficult. It is assumed that the AA level in seminal plasma reflects its level in the seminiferous tubules [28], but no experimental evidence supports this relationship. Thus, two pools of AA exist in the mammalian reproductive system, one in the testis and epididymis, and another in the accessory glands. The first pool may have a protective effect on sperm cells during spermatogenesis and maturation. However, further studies are necessary to support this hypothesis. A protective effect of AA can be extended to somatic and glandular testicular tissue as well, for example, in synthesis of collagen or steroidogenesis, and may also be responsible for the overall dietary effect of AA observed in our experiment. The size of the testicular and epididymal AA pool is difficult to evaluate, and it is estimated indirectly from seminal plasma AA concentrations. The pool originating from the seminal vesicles and comprising most of the AA from seminal plasma can affect spermatozoa in vivo only after ejaculation. A protective role of this AA pool for the sperm until fertilization has been suggested [28]. Accessory glands are absent in rainbow trout, and secretory activity of the spermiduct epithelium may contribute to seminal plasma [29]. It can be assumed that in this species, seminal plasma AA concentrations represent those in the testes lobules and duct. For this reason, this fish can be useful in studies of long-term effects of seminal plasma AA on sperm physiology. Because the reproductive cycle of rainbow trout is well defined and spermatogenesis is seasonal, it opens unique possibilities for detailed studies of AA action through the reproductive cycle.

Substantially more time is needed to reduce tissue AA levels in poikilothermic fish than in homeothermic mammals. Chinoy et al. [14] observed scorbutic symptoms in guinea pigs after three weeks of experimental treatment. A number of sperm parameters underwent a dramatic decline after this period. Fraga et al. [28] observed about a 70% decline in the AA concentration of human seminal plasma after two months of a low-AA diet (5–20 mg/day). In our study, we observed 4.74 ppm vitamin C in seminal plasma of the AA-free treatment group at the beginning of the reproductive season, which indicates that some vitamin was conserved even after five months of feeding on diets without AP. This could be a reason why most parameters of milt (including biochemical ones, Ciereszko at al., manuscript in preparation) of the AA-deficient group were similar to those of the AA-supplemented groups during milt production at the beginning of spawning season. AA in fish seminal plasma is well protected against oxidation. After two weeks of storage of rainbow trout semen (which has a pH of 7.8–8.0) on ice with daily flushing of oxygen, more than half the AA pool was preserved [15], although it is known that the vitamin in the alkaline environment of buffers is

destroyed within hours. Recently, we have obtained evidence that the protective effect of rainbow trout seminal plasma is exerted by its protein fractions of high molecular weight [30]. It is clear that frequent milt collection led to quick exhaustion of the seminal pool of vitamin C in the deficient fish and to a decline of the vitamin level in AP-supplemented groups. The AA concentration could not be fully recovered by dietary AA, most likely because of lower food intake by fish during spawning and because of low water temperatures. The low vitamin C concentration at the end of reproductive season coincides with lower sperm quality at this time [23].

Rainbow trout spermatozoa are motile for only about 30 sec after being released into water. They can cover a distance of only 2–3 mm during this time [23, 31], after which they become immotile because of ATP exhaustion [32] and hypoosmotic shock. The rainbow trout ovum is large, having a diameter of up to 6 mm. There is only one site in teleost fish eggs for spermatozoon penetration, the micropyle at an animal pole. The probability for reaching the micropyle and subsequent fertilization depends on the density of motile sperm surrounding the egg. It was established that about 30 000–500 000 spermatozoa per egg is necessary for successful fertilization under conditions of artificial insemination [23]. Those numbers can be higher during natural spawning. Since we found that both sperm motility and concentration were impaired by AA deficiency, we suggest that vitamin C deficiency diminishes fertility of rainbow trout males. The findings that vitamin C deficiency reduced the ability of sperm to fertilize eggs (Dabrowski and Ciereszko, unpublished data) and sperm motion as evaluated by computer-assisted motion analysis (Toth et al., unpublished data) support this conclusion.

The mechanism by which AA protects sperm quality is not known. An antioxidant function of the vitamin has mainly been postulated. Data of Singh et al. [33] suggest that vitamin C can lower lipid peroxidation of buffalo sperm membranes. Beconi et al. [34] also reported lower malonaldehyde production by frozen bovine sperm in the presence of AA. The protective effect of AA on sperm membranes was responsible for this phenomenon according to the authors. Tarin and Trounson [35] found that AA protected mouse embryos during freezing-thawing, which they interpreted as a result of diminished lipid peroxidation. Since lipid peroxidation has been proven detrimental to sperm motility [36–38], it is probable that the lower motility in AA-depleted milt samples could be attributed to a higher amount of lipid peroxides in the sperm membranes. However, special care will be necessary in further studies aimed at obtaining direct evidence of the protective function of AA against cellular peroxidation, since vitamin C can have pro-oxidant properties in vitro [30, 39]. Fraga et al. [28] demonstrated that the sperm genome can also be protected by vitamin C. They found that the amount of oxidized nucleoside 8-hydroxy-2′-deoxyguanosine, one of more than twenty major products of oxidative damage to DNA, in human spermatozoa is related to the AA level in human seminal plasma. The authors also demonstrated that its concentration increased after depletion of AA in seminal plasma and in the diet and, furthermore, that it was reduced after dietary repletion of the vitamin. Whether seminal AA protects the fish sperm genome is currently being studied in our laboratory.

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